PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C12Q 1/68, C12N 15/00 C07H 15/12 | A1 | (11) International Publication Number: WO 90/00624

(43) International Publication Date: 25 January 1990 (25.01.90)

(21) International Application Number: PCT/US89/02884

(22) International Filing Date: 30 June 1989 (30.06.89)

(30) Priority data: 215,135 5 July 1988 (05.07.88) US

(71) Applicant: BAYLOR COLLEGE OF MEDECINE [US/US]; One Baylor Plaza, Houston, TX 77030 (US).

(72) Inventor: LUPSKI, James, R. ; Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030 (US).

(74) Agent: PAUL, Thomas, D.; Fulbright & Jaworski, 1301 McKinney, Houston, TX 77010 (US).

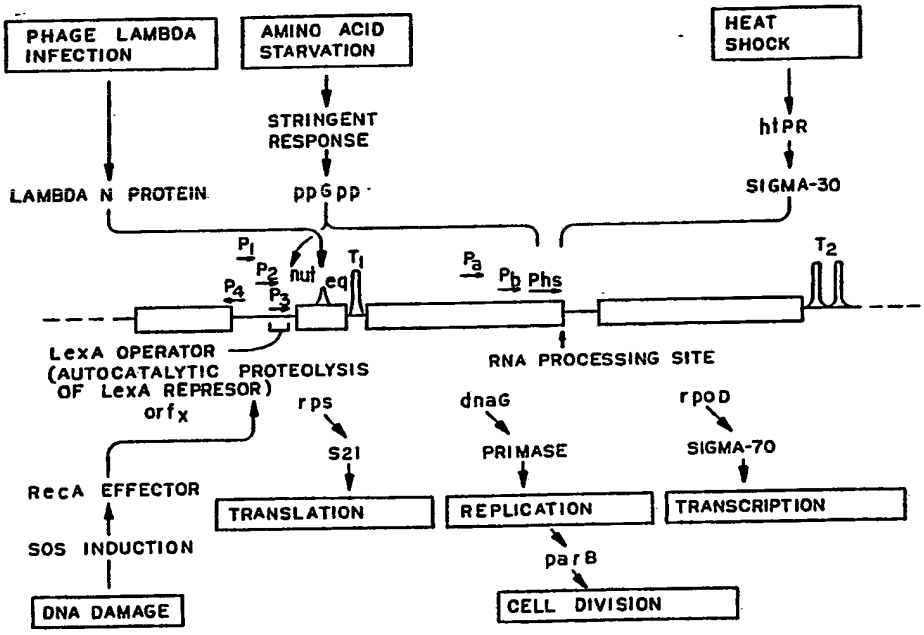
(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ANTISENSE OLIGONUCLEOTIDE ANTIBIOTICS COMPLEMENTARY TO THE MACROMOLECULAR SYNTHESIS OPERON, METHODS OF TREATING BACTERIAL INFECTIONS AND METHODS FOR IDENTIFICATION OF BACTERIA

(57) Abstract

A method of interrupting the expression of a macromolecular synthesis operon in bacteria comprising the step of binding an antisense oligonucleotide to a single stranded DNA or to a mRNA transcribed from the macromolecular synthesis operon. The antisense oligonucleotide can be either sequence specific to a unique intergenic sequence or a sequence specific to a bacterial homologous sequence. By interrupting the expression of the macromolecular synthesis operon bacterial infections can be treated. Examples of antisense oligonucleotides are 5'CATCCAAGCAGTGGTAAAACTGTTT 3', 5'TCACCGATCGGCGTTTCCA 3', 5'GGCCCCGATTTTAGCAA 3', 5'CTTGCGTAAGCGCCGGGGG 3', and 5'TATTCGATGCTTTAGTGC 3'. The ability of the antisense oligonucleotide to bind the mRNA or single stranded DNA also allows the identification of the bacteria by using a unique intergenic antisense oligonucleotide to bind to the single stranded DNA or to the mRNA transcribed form the macromolecular synthesis operon. A method for competitively inhibiting the protein products of the MMS operon with oligonucleotides is also disclosed.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CM	Cameroon
DE	Germany, Federal Republic of
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
HU	Hungary
IT	Italy
JP	Japan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
MC	Monaco
MG	Madagascar
ML	Mali
MR	Mauritania
MW	Malawi
NL	Netherlands
NO	Norway
RO	Romania
SD	Sudan
SE	Sweden
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
US	United States of America

ANTISENSE OLIGONUCLEOTIDE ANTIBIOTICS COMPLEMENTARY TO THE MACROMOLECULAR SYNTHESIS OPERON, METHODS OF TREATING BACTERIAL INFECTIONS AND METHODS FOR IDENTIFICATION OF BACTERIA

FIELD OF THE INVENTION

The present invention relates generally to antisense oligonucleotides which bind to a messenger RNA. More particularly it relates to antisense oligonucleotides which bind to messenger RNA transcribed from the macromolecular synthesis operon of bacteria. It also relates to the treatment of bacterial infections by the introduction of antisense oligonucleotides into bacteria. It further relates to the method of identification of bacteria by the binding of an antisense oligonucleotide specifically to a unique sequence in the intergenic regions of the macromolecular synthesis operon of bacteria. It also relates to the treatment of bacterial infections by competitive inhibition of the macromolecular synthesis operon gene products by utilizing oligonucleotides known to act as recognition sequences for the MMS operon protein products.

BACKGROUND OF THE INVENTION

It has been demonstrated that the genes involved in initiating the synthesis of DNA, RNA and protein in bacteria are contained in one single structural unit named the macromolecular synthesis operon (MMS). The genes are part of a single transcription unit and have been identified as rpsU encoding ribosomal protein S21 involved in initiating translation, dnaG encoding the protein primase which initiates DNA replication and rpoD which encodes sigma-70 involved in initiating transcription. The operon structure is found in both gram negative bacteria, such as Escherichia coli and Salmonella typhimurium, and in gram positive bacteria such as Bacillus subtilis. The individual structural genes are conserved and have large areas of homology. On the other hand, the intergenic sequences between the structural gene within the operon are unique to each bacterial species. The MMS operon appears to be a central information processing unit for directing the flow of genetic information. The organization of the operon suggests that under certain physiological conditions there is a need for coordination of synthesis of the information macromoleculas (DNA, RNA and protein) in the cell and hence a coregulation of the initiator genes. Since the synthesis of each class of macromolecule appears to be regulated at its initiation step, regulation of the MMS operon most likely plays a role in regulating cell growth.

The MMS operon contains three structural genes. The rpsU gene encodes the ribosomal protein S21 which is required for specific initiation of messenger RNA (mRNA) translation. The protein S21 interacts with a stretch of ribosomal RNA (rRNA) complementary to the mRNA ribosomal binding site called the Shine-Dalgarno sequence located at the 3' end of the 16S rRNA. Colicin E3 removes 50

nucleotides from the 3' terminus of 16S rRNA. E3 treated ribosomes cannot carry out polypeptide chain initiation nor chain elongation. In reconstitution experiments, E3 treated ribosomes bind all 30S proteins except S21. RNA protein cross-linking experiments demonstrate that protein S21 is cross-linked to the 3' dodecanucleotide of the 16S rRNA. The base-pairing potential of the 3' terminus of 16S rRNA depends on the functional state of the 30S subunit and the presence of S21, which is required for specific initiation of E. coli and phage MS2 mRNA translation.

Initiation of DNA replication requires a priming RNA which is synthesized by the dnaG gene product, primase. This protein binds to the phage G4 origin of replication. Primase also is known to interact with the multienzyme complex primosome to initiate synthesis of Okazaki fragments on the chromosomal replication fork-lagging strand of E. coli. Primase is the sole priming enzyme required for initiation of DNA replication at the origin of the E. coli chromosome. A parB mutation in the dnaG gene results in abnormal partition of chromosomes and was originally isolated as a thermosensitive mutant affecting DNA synthesis and cellular division. Thus, in addition to initiation of DNA replication, the dnaG gene appears to play some role in regulating cell division.

The rpoD gene product sigma-70 is involved in the recognition of promoter sequences for the specific initiation of RNA transcription. Sigma-70 interacts with the core polymerase $\alpha_2\beta\beta'$ conferring specificity for promoter sequences. Sigma-70 is a member of a large family of RNA polymerase sigma factors. Thus, the macromolecular synthesis operon gene products share a common mechanism. Through protein-nucleic acid interactions the gene products of the MMS operon bind

specific nucleotide sequences. For example S21 binds the Shine-Dalgarno sequence/ribosome binding site, primase binds the origin of replication, and sigma-70 binds a promoter sequence. These interactions result in initiation of synthesis of protein, DNA or RNA respectively.

Antisense RNAs have been utilized both in nature and experimentally to regulate gene expression. For example antisense RNA is important in plasmid DNA copy number control, in development of bacteriophage P22. Antisense RNAs have been used experimentally to specifically inhibit in vitro translation of mRNA coding from Drosophila hsp23, to inhibit Rous sarcoma virus replication and to inhibit 3T3 cell proliferation when directed toward the oncogene c-fos. Furthermore, it is not necessary to use the entire antisense mRNA since a short antisense oligonucleotide can inhibit gene expression. This is seen in the inhibition of chloramphenicol acetyltransferase gene expression and in the inhibition of specific antiviral activity to vesicular stomatitus virus by inhibiting the N protein initiation site. Antisense oligonucleotides to the c-myc onocogene have been demonstrated to inhibit entry into the S phase but not the progress from G_0 to G_1. Finally, inhibition of cellular proliferation has been demonstrated by the use of antisense oligodeoxynucleotides to PCNA cyclin.

Antibiotics are important pharmaceuticals for the treatment of infectious diseases in a variety of animals including man. The tremendous utility and efficacy of antibiotics results from the interruption of bacterial (prokaryotic) cell growth with minimal damage or side effects to the eukaryotic host harboring the pathogenic organisms. All antibiotics destroy bacteria by interfering with the normal flow of genetic information.

This is performed by inhibition of any one of the following: DNA replication, that is, DNA to DNA (for example, the drugs Novobiocin and Nalidixic acid); transcription, that is, DNA to RNA (for example, Rifampin); translation, that is, RNA to protein (for example, tetracyclines, erythromycin and kamanycin); or cell wall synthesis (for example, penicillins).

The present invention provides a new class of antibiotics and a method for the treatment of bacterial infections either generally or specifically. The antibiotics are antisense oligonucleotide sequences which bind mRNA transcribed from the MMS operon. This is a new method of treating bacterial infections by interfering with the fundamental structural unit that regulates the growth and replication of bacteria.

SUMMARY OF THE INVENTION

An object of the present invention is the provision of a method for the treatment of bacterial infections.

An additional object of the present invention is the use of antisense oligonucleotides to treat bacterial infections.

A further object of the present invention is a method for identifying bacteria.

An additional object of the present invention is the provision of antibiotics which interrupt the operation of the macromolecular synthesis operon in bacteria.

A further object of the present invention is the use of competitive inhibitors to interfere with the nucleotide recognition site of the macromolecular operon gene products.

Thus, in accomplishing the foregoing objects there is provided in accordance with one aspect of the present invention a method of interrupting the expression of a MMS operon comprising the step of binding an

antisense oligonucleotide to a mRNA transcribed from said MMS operon. The antisense oligonucleotide sequence can be specific to a unique intergenic sequence in the mRNA or it can be a sequence which is specific to a region of the mRNA containing a sequence which is homologous between bacterial strains or any combination of these.

A further aspect of the present invention is the method for treating bacterial infections by interrupting the expression of the MMS operon by binding an antisense oligonucleotide antibiotic to a mRNA transcribed from the MMS operon.

In preferred embodiments, the antisense oligonucleotide antibiotic can be selected from the following sequences:

5' CATCCAAAGCAGTGGTAAAACTGTTT 3' (AOAMMS-dnaG),

5' TCACCGATCGGCGTTTCCA 3' (AOAMMS-rpoD),

5' GGCCCCGATTTTTAGCAA 3'(AOAMMS-Eco);

5' CTTGCGTAAGCGCCGGGG 3' (AOAMMS-Sty) and

5' TATTCGATGCTTTAGTGC 3'(AOAMMS-Bsu).

Another aspect of the present invention is a method for typing or identifying bacteria comprising the steps of binding a unique intergenic antisense oligonucleotide to a mRNA transcribed from the MMS operon and then determining the amount of binding between the species specific MMS oligonucleotide and the mRNA transcribed from the MMS operon of a given bacterial species.

In the treatment of a bacterial infection or in the identification of bacteria the antisense oligonucleotide is at least 10 nucleotides (10 mer). In a preferred embodiment, an oligonucleotide of 16 to 26 mers is used.

An additional aspect of the present invention is the provision of an antisense oligonucleotide antibiotic of at least 10 nucleotides, wherein said oligonucleotide

binds to a mRNA transcribed from a MMS operon. In one embodiment the antibiotic further comprises a carrier molecule linked to the oligonucleotide for facilitating the uptake of the oligonucleotide into the bacterium. The carrier molecule can be an amino acid, and in one preferred embodiment is leucine. In another embodiment the 3' end of the oligonucleotide is derivatized to prevent the degradation, e.g. by exonucleases, of the oligonucleotide after bacteria uptake.

Other and further objects, features and advantages will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure when taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the MMS operon shown in schematic form. It contains three genes, one each, involved in the initiation of translation (rpsU), replication (dnaG) and transcription (rpoD).

Figure 2 depicts the regulation of the E. coli MMS operon. The three genes in the MMS operon are depicted as closed boxes. The cis-acting regulatory sequences include promoters (P_x, P_1, P_2, P_3, P_a, P_b, P_{hs}), terminators (T_1 and T_2), a LexA binding site, nut_{eq} and an RNA processing site. The trans acting factors are shown with arrows drawn to where they are believed to act. The NusA protein increases rpoD gene expression, but its site of action is unknown. Global regulatory networks that interact with the MMS operon include the SOS, heat shock and stringent response. A functional role for orf_x has not been assigned, but the proximity of P_x and the conservation of the orf_x sequences in E. coli and S. typhimurium suggests a possible MMS operon regulatory role. There are several other open reading frames further upstream with no

assigned function and the nearest gene mapped on the E. coli chromosome is the cca gene which is 14 kb away.

Figure 3 is a comparison of the MMS operon in different species. The structure of the MMS operon has been determined for E. coli, S. typhimurium and B. subtilis. The genes are depicted by open boxes with the size given in base pairs (bp) including termination codon. The size of the intergenic sequences is given below. Position of promoters (P) are denoted. AOAMMS - Eco is complementary to the E. coli MMS operon rpsU-dnaG intergenic sequences. AOAMMS - Sty is complementary to the S. Typhimurium MMS operon rpsU-dnaG intergenic sequences. AOAMMS - Bsu is complementary to the B. subtilis MMS operon rpsU-dnaG intergenic sequences.

Figure 4 shows a 5' modified antisense oligonucleotide antibiotic containing the addition of leucine.

Figure 5 shows a 3' modified antisense oligonucleotide antibiotic.

Figure 6 shows the homologies between bacterial strains for the primase gene. The information was generated from DNA sequences in GenBank utilizing the Molecular Biology Information Resources Multialign program to optimize homology searches of protein sequence data. The data is aligned from left to right on the abscissa, the amino terminal to the carboxy terminal portions of the protein. The numbers represent the amino acid positions in the protein primary sequence. In (a) B. subtilis was compared to E. coli, while in (b) S. typhimurium was compared to E. coli, and in (c) B. subtilis is compared to S. typhimurium. In (d), the S. typhimurium and B. subtilis primase protein sequences have been aligned to the E. coli dnaG primase in the amino terminal region. Upper case letters represent aligned non-identical amino acids while lower case letters signify non-aligned amino

acids. The dashes represent aligned identical bases while the dots signify gaps. The data demonstrate that the primase proteins are related and share homology domains particularly in the amino terminal regions. The nucleotide sequence encoding these areas of amino acid homology are also very homologous.

Figure 7 is a picture of 1% agarose gel showing antisense binding.

The drawings are not necessarily to scale and certain features of the invention may be exaggerated in scale or shown in schematic form in the interest of clarity and conciseness.

DETAILED DESCRIPTION

It will be readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

The macromolecular synthesis (MMS) operon includes genes involved in initiating translation, rpsU replication, dnaG, and transcription, rpoD. These genes are contained within a single transcriptional unit, Figures 1 and 2, and are involved in initiating synthesis of the major information macromolecules of the cell. The organization of the operon suggests that under certain physiological conditions there is a need for coordination of synthesis of DNA, RNA and protein in the cell and hence a coregulation of the initiator genes. Since the synthesis of each class of information macromolecule (DNA, RNA and protein) appears to be regulated at its initiation step, regulation of the MMS operon most likely plays a role in regulating cell growth.

In the MMS operon cis-acting regulatory sequences can occur within the coding regions. In gram-negative bacteria these include the nut_{eg} site within the rpsU structural gene and promoters P_a, P_b, and P_{hs} in the

dnaG structural gene. Promoter P_3 of the B. subtillis MMS operon is within this gene coding for P23. Other cis-acting regulatory sequences are located in the intergenic regions; terminator T_1 is located between rpsU and dnaG and an RNA processing site occurs in the dnaG-rpoD intergenic sequences. Thus, multiple cis-acting regulatory sequences allow discoordinate regulation as well as differential relative rates of individual gene expression within this operon structure.

Codon usage can affect relative amounts of individual gene expression. The presence of codon preference reflects the relative concentrations of isoaccepting tRNA species in the cell. The use of rare codons provides a means to ensure low level expression of regulatory genes. The dnaG gene contains greater than ten times the number of rare triplet codons as other E. coli genes and the absolute number of rare codons in the dnaG mRNA is similar to that of other control genes (e.g. lacI, trpR). Rare codons also occur in the S. typhimurium dnaG mRNA and the dnaE gene of B. subtilis. An additional translational regulatory mechanism operative in the MMS operon relies on the occurrence of ribosome binding sites with varying degrees of complementarity to the Shine-Dalgarno sequence. This can be seen in the E. coli dnaG gene, and is presumably due to the difference in free energy of binding leading to less efficient binding of the ribosome to the dnaG portion of the MMS mRNA. Both of these translational regulatory mechanisms, rare codon usage and altered ribosome binding affinity may partially explain the observed apparent discoordination of expression of the genes in this operon. The steady state relative abundances for the MMS operon protein products in the E. coli cell are 40,000 for S21, 50 for primase and approximately 3000 for sigma-70.

Comparative analysis of three sequenced MMS operons reveals several interesting features (Figure 3). All of the operons contain three open reading frames and transcription of the operons is initiated by several promoters at the 5' end. The major promoters have overlapping nucleotide sequences (-10 and -35 regions) and the cis-acting regulatory sequences appear to be clustered in small regions. Each operon contains a heat shock promoter (P_{hs}) within the DNA replication initiation gene, dnaG or dnaE. The E. coli and S. typhimurium operons contain an open reading frame (orf_x) upstream of the external promoters (P_1, P_2, P_3). Only 7 bp separate the -35 sequences of P_x and P_1 in E. coli while these sequences actually overlap in the S. typhimurium operon.

The central gene in the MMS operon is the one involved in initiating DNA replication. The dnaG gene product, primase has several activities which include (i) a protein-protein interaction with the primosome complex, (ii) a protein-nucleic acid interaction for recognition of the origin, (iii) an RNA polymerase activity to synthesize the primer RNA and (iv) a role in the partitioning of chromosomes as suggested by the parB mutation in the dnaG gene. There are no promoters which transcribe the dnaG gene directly. A 5' transcription terminator, poor ribosome binding site, occurrence of rare codons and clustering of rare codons are all mechanisms that maintain low level expression of this gene. Overexpression of the dnaG gene from a regulated promoter on an autonomously replicating plasmid kills the host cells. Evidence that regulation of dnaG expression directly affects cell growth comes from Tn5 mutagenesis data. A cloned dnaG gene with the MMS operon promoters intact, on a multicopy plasmid slows the growth rate of the host cell harboring it. After insertion of Tn5 into the dnaG promoter regions,

presumably leading to decreased dnaG gene expression, growth rates return to control levels demonstrating that an increased dnaG expression can affect growth. Isolation of the parB mutation also suggests a direct role for dnaG in chromosome partitioning, cell division, and therefore, bacterial cell growth. The primase proteins encoded by the DNA replication initiation genes from the three sequenced MMS operons contain several regions of homology (Figure 6).

The MMS operon is under very complex regulatory control which, teleologically would be expected of a unit whose control is important to regulation of cell growth. In addition to the intrinsic complex regulation, the operon interacts with several global regulatory networks including heat shock, the stringent response, and SOS. This operon appears to have evolved ways to be regulated both as a single unit and as a group of independent units by strategic positioning of transcriptional and translational control signals. The fact that the operon is the same in E. coli and S. typhimurium and very similar in B. subtilis suggests there is a selective advantage to evolving such a structure.

The term "oligonucleotide" as used herein defines a molecule comprised of more than three deoxyribonucleotides or ribonucleotides. Its exact length will depend on many factors relating to the ultimate function or use of the oligonucleotide.

The term "homologous sequence" as used herein defines a sequence within the MMS operon which has been conserved in bacterial species such that the sequence is nearly identical among a variety of species. Thus, this sequence because of its identity cannot be used to distinguish different types of bacteria from themselves but can be used as a location which can be attacked by a single agent to interfere with a variety of bacterial species.

The term "unique intergenic sequences" as used herein defines a section of non-coding DNA between specific genes. In the MMS operon the intergenic sequences as seen in Figure 3 are unique for each different strain of bacteria. Thus, a specific sequence will be characteristic for a specific strain of bacteria and thus, can be used to identify the bacteria or for the specific binding an an agent to kill or interrupt the functioning of that type of bacteria only.

The term "antisense" as used herein defines an oligonucleotide the sequence of which is complementary to the sense strand of the MMS operon. An antisense oligonucleotide will bind (form a complex by Watson-Crick base pairing) in a complementary fashion to the messenger RNA molecule which has been transcribed from the MMS operon, as well as to a single stranded DNA of the MMS operon.

The term "antibiotic" as used herein means an oligonucleotide capable of interfering with the MMS operon to slow down bacterial growth thereby arresting growth and provoking cell death.

"Derivitizing" the oligonucleotide means altering the structure of the oligonucleotide to perform a specific function (e.g. (1) an addition to the 5' end to afford uptake into the cell; (2) blocking the 3' end to prevent exonucleolytic breakdown). This procedure provides a more functional and stable oligonucleotide when it is in the bacteria. For example, the 3' end can be derivitized by adding a phosphorothioate linked nucleotide.

In one embodiment of the present invention there is included a method of interrupting the expression of a MMS operon comprising the step of binding antisense oligonucleotide to an mRNA transcribed from the MMS operon. In this method the antisense oligonucleotide binds to the mRNA which is transcribed from the MMS

operon. After the binding of the antisense oligonucleotide the mRNA is unable to be translated into the proteins encoded by the MMS operon. In order to inactivate the mRNA, only a small segment of the mRNA must be bound to the antisense oligonucleotide.

The antisense oligonucleotide is selected from the group consisting of a sequence specific to a unique intergenic sequence, a sequence specific to a bacterial homologous expressed sequence and any combination thereof.

By binding to a specific unique intergenic sequence encoded in the single stranded DNA or mRNA which has been transcribed from the MMS operon, the antibiotic can be targeted to interrupt and kill the specific type of bacteria. By binding to the homologous sequence, the antibiotic can be targeted to a wide variety of bacteria all containing the homologous sequence. Depending on the length of the oligonucleotide or the location of the mRNA which is bound, the oligonucleotide may overlap and bind to both a unique sequence and a homologous sequence.

Although the length of the oligonucleotide which is necessary to inhibit the functioning of the mRNA is unknown, it should be at least 10 nucleotides (10 mer). In one embodiment of the present invention, the oligonucleotide is in the range of 16 to 26 mers.

An additional aspect of the present invention is a method for treating bacterial infections comprising the step of interrupting the expression of a MMS operon by binding an antisense oligonucleotide antibiotic to a mRNA transcribed from said MMS operon. The antisense oligonucleotide antibiotic can bind to either a homologous sequence, a unique intergenic sequence or a combination thereof. Some examples of sequences which can be used to bind to the mRNA to interrupt the function of the MMS operon and thus to treat bacterial infections are seen in Table 1.

Table 1

Sequences which bind to mRNA transcribed from the MMS operon

(1) 5'CATCCAAAGCAGTGGTAAAACTGTTT 3' (AOAMMS-dnaG),

(2) 5'TCACCGATCGGCGTTTCCA 3' (AOAMMS-rpoD),

(3) 5' GGCCCCGATTTTTAGCAA 3' (AOAMMS-Eco),

(4) 5' CTTGCGTAAGCGCCGGGG 3' (AOAMMS-Sty),

(5) 5' TATTCGATGCTTTAGTGC 3' (AOAMMS-Bsu).

The first two sequences (1-2) bind to bacterial homologous sequences and thus are not specific to any type of bacteria. These sequences can be used to treat a wide class of bacterial infections since they attack both gram positive and gram negative bacteria. The last three sequences (3-5) are unique intergenic sequences which bind to specific sequences in specific bacteria. For example sequence (3) is specific to E. coli. Thus, employing this antisense oligonucleotide antibiotic will specifically inhibit the MMS operon of E. coli while not attacking the MMS operon of other bacteria. Sequence (4) specifically binds the transcribed mRNA of S. typhimurium and sequence (5) specifically binds the mRNA of B. subtilis. Thus, by employing the antisense oligonucleotide antibiotics (3-5) a specific antibiotic can be used to kill a specific bacteria. Thus, the treatment to kill or interfere with the reproduction of specific bacterial strains can be targeted.

In the preferred embodiment, using unique sequences, the nucleotide sequence of the proposed antisense oligonucloetide antibiotics is complementary to the intergenic region of the 5' side of the DNA replication initiation gene (dnaG or dnaE) (see Figure 3). This region of the MMS operon is chosen because the replication initiation gene has the lowest level of expression within the operon. Furthermore, in E. coli and S. typhimurium, this gene is located downstream from a

terminator and is not directly transcribed by any promoter. In order to provide a more stable ineraction with the mRNA the primary sequences of the antisense oligonucleotide antibiotic are chosen to maximize GC base pairing. However, there is usually a balance between maintaining the uniqueness of the sequence and maximizing the GC base pairing.

Another embodiment of the invention is a method of identifying bacteria comprising the steps of binding a unique species specific intergenic antisense oligonucleotide to a mRNA transcribed from a MMS operon of a given species and determining the amount of said binding. The unique sequence will only bind to a specific bacteria strain, therefore no binding indicates a different strain and binding indicates the strain with the specific sequence. Each bacteria strain contains its own unique intergenic sequence which can be used to uniquely identify each strain. The mRNA which is transcribed from the MMS operon spans the whole operon and contains the unique intergenic sequence. By designing oligonucleotides which bind to these unique sequences, the diagnosis and treatment can be tailored to only interfere with the functioning of a MMS operon in those bacteria strains which have that unique sequence. Thus, by using a variety of antisense oligonucleotide probes, bacteria can be typed for each individual strain. The amount of binding can be determined by a variety of methods known to those skilled in the art, including radioisotopes, enzymes, fluorescers, antibodies and chemiluminescers For example, the unique species specific intergenic antisense oligonucleotides can be labelled with biotin and then identified by a Strep avidin complex or a fluorescent tag.

For example, the antisense oligonucleotide of sequence (3) table 1 can be used to identify E. coli, whereas the antisense oligonucleotide of sequence (4)

table 1 can be used to identify S. typhimurium and the antisense oligonucleotide of sequence (5) table 1 can be used to identify B. subtilis. One skilled in the art will readily recognize that as additional MMS operon intergenic sequences are sequenced additional bacteria can be identified by antisense oligonucleotides synthesized to the unique intergenic sequences.

In bacteria typing the length of the antisense oligonucleotide will be determined by the size necessary to bind specifically to the unique sequence. The oligonucleotide will be at least 10 nucleotides. In one preferred embodiment the sequences are between 16 and 26 mers. Examples of some preferred sequences are found in table 1 sequences (3-5).

In order for the antisense oligonucleotide antibiotic to effectively interrupt the MMS operon function by binding to the mRNA transcribed from the MMS operon, the antisense oligonucleotide antibiotic must enter the bacterial cell. Although some oligonucleotides can be taken up by certain bacterial cells (e.g. Haemophillus), other oligonucleotides will need to be modified to facilitate uptake. Thus, it may be necessary to link a carrier molecule, for example an amino acid, to the oligonucleotide. In Figure 4, the oligonucleotide is modified at the 5' end by adding a leucine molecule to the oligonucleotide. Bacteria have multiple transport systems for the recognition and uptake of molecules of leucine. The addition of this amino acid to the oligonucleotide will facilitate the uptake of the oligonucleotide in the bacteria and will not interfere with the binding of the antisense oligonucleotide to the mRNA molecule.

One skilled in the art will readily recognize that other methods are available for facilitating the uptake of the antisense oligonucleotide antibiotic in the bacteria. For example, addition of other amino acids will

enable utilization of specific amino acid transport systems. Addition of lactose to the oligonucleotide by a covalent linkage may enable transport by lactose permease (product of the lac operon Y gene). Other sugar transport systems, known to be functional in bacteria, can be utilized to facilitate uptake into the bacterial cell.

Once an oligonucleotide with or without the carrier has entered the bacterial cell, it is important that it remain stable for the time period necessary to bind to the mRNA transcribed by the MMS operon. In one embodiment of the present invention, the oligonucleotide is derivatized at the 3' end to prevent degradation of the oligonucleotide (Figure 5). Other methods are known to alter the 3' and/or 5' ends of oligonucleotides to prolong the intracellular life and thus increase the availability for binding to the mRNA.

In addition to interrupting the MMS operon by binding to the mRNA transcribed from the operon, it is also possible to control other downstream products of the MMS operon to interrupt bacteria and to treat bacterial infections. For example, interrupting the function of the proteins encoded in the MMS operon will also interrupt the function of the MMS operon and lead to death of the bacteria.

One embodiment of the present invention is a method for treating bacterial infections comprising the step of interrupting the function of proteins selected from the group consisting of S21, primase and sigma-70. This method comprises the step of competitively inhibiting a recognition site of a protein encoded by the MMS operon by introducing a competitive oligonucleotide into the bacteria.

The S21 recognition site includes the Shine-Dalagarno sequence located at the 3' end of the 16S rRNA and may be inhibited by introducing an

oligonucleotide which competitively inhibits the binding of S21 in the bacteria. For example, an oligonucleotide of the sequence 5'GATCACCTCCTTA 3' which is the 3' end of the 16S rRNA (the Shine-Dalagarno sequence).

The primase recognition site includes the phage G4 origin of replication site. Thus by introducing into bacteria a competitive oligonucleotide which interfers with this recognition site, bacterial growth and survival may be inhibited. An example of this competitive inhibitor is 5'GGCCGCCCCACATTGGGCAGGTATCTGACCAGTAGAGGGGCGGCC 3' which is the loop III of the bacteriophage G4 ori_c.

The sigma-70 recognition site includes the core polymerase α_2BB' and this interaction confers specificity for promoter sequences. An example of this competitive inhibitor is 5'TTGACATAAATACCACTGGCGGTGATACT 3'. This sequence is the bacteriophase lambda P_L promoter. This is the strongest promoter in E. coli and thus has the strongest known binding with RNA polymerase.

Thus the introduction of competitive oligonucleotides for these sequences into the bacteria will result in competitive interaction with the protein recognition site, thus preventing the binding of the S21, primase or sigma-70 molecules to the recognition site. This will interrupt normal cell function, growth and replication. Introduction of these oligonucleotides into the bacteria, disrupts the MMS operon's function and thus successfully treats bacterial infections.

Example I

To inhibit cell growth, an inoculum of E. coli and B. subtilis are mixed in a single test tube and an antisense oligonucleotide to E. coli (AOAMMS-Eco) is added to the cell inoculum. The culture is gram strained after several hours of growth. Gram positive organisms are seen and there is a paucity of gram negative organisms. In a

corollary experiment, an antisense oligonucleotide to B. subtilis (AOAMMS-Bsu) is added to a mixed inoculum of E. coli and B. subtlis and it is grown for several hours. On subsequent gram strain there is found negative rods. These experiments demonstrate species specific antisense oligonucleotide demise of bacterial organisms.

EXAMPLE II

To show that the expressed sequences within the MMS operon (rpsU, dnaG, rpoD) contain conserved homologous DNA sequences, the following oligonucleotide which recognized conserved DNA sequences within the dnaG gene.

AOAMMS - dnaG, 5'- CATCCAAAGCAGTGGTAAAACTGTTT-3' was synthesized: (sequence 1, Table 1)

This oligonucleotide was end labeled and used as a probe in Southern blotting. DNA was isolated from 12 different pathogenic strains of Salmonella obtained from the body fluids of infected patients, digested with HindIII and run on a 1% agarose gel. This digested chromosomal DNA was probed with the end-labeled dnaG oligonucleotide AOAMMS.

As seen in Figure 7, there is conservation of the oligonucleotide AOAMMS - dnaG in different pathogenic strains of Salmonella. The Southern blot shows homology of the oligonucleotide AOAMMS-dnaG to a laboratory control strain of Salmonella (LT-2) (lane 1) and twelve (12) different pathogenic strains isolated from body fluids of patients (lanes 2-13). There was no hybridization to human DNA (the negative control on lane 14), and as a positive control; a plasmid containing the DNA sequences in the probe showed a hybridization signal (lane 16). Lane 15 has lambda DNA cut with Hind III as a marker. On the far right are the sizes in kilobase pairs as determined on the agarose gel before Southern transfer.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out

the objects and obtain the ends and advantages mentioned, as well as, those inherent therein. The oligonucleotides, antibiotics, compounds, methods, procedures and techniques described herein are presently representative of preferred embodiments, are intended to be exemplary, and are not intended as limitations on the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention or defined by the scope of the appended claims.

WHAT IS CLAIMED IS:

CLAIMS

1. A method of interrupting the expression of a MMS operon, comprising the step of binding an antisense oligonucleotide to a mRNA transcribed from said MMS operon.

2. The method of claim 1, wherein the antisense oligonucleotide is selected from the group consisting of a sequence specific to a unique intergenic sequence, a sequence specific to a bacterial homolgous sequence and any combination thereof.

3. The method of claim 2, wherein the antisense oligonucleotide is at least 10 mers.

4. The method of claim 3, wherein the antisense oligonucleotide is 16 to 26 mers.

5. A method for treating bacterial infections comprising the step of interrupting the expression of a MMS operon by binding an antisense oligonucleotide antibiotic to a mRNA transcribed from said MMS operon.

6. The method of claim 5, wherein the antisense oligonucleotide antibiotic binds to a bacterial homologous sequence in the mRNA transcribed from said MMS operon.

7. The method of claim 6, wherein the antisense oligonucleotide antibiotic is selected from the group consisting of 5'CATCCAAAGCAGTGGTAAAACTGTTT 3' and 5'TCACCGATCGGCGTTTCCA 3'.

8. The method of claim 5, wherein the antisense oligonucleotide antibiotic binds to an intergenic sequence, said intergenic sequence is unique for each strain of bacteria.

9. The method of claim 8, wherein the antisense oligonucleotide antibiotic is selected from the group consisting of:

5' GGCCCCGATTTTTAGCAA 3' which binds to the transcribed mRNA of E. coli, 5' CTTGCGTAAGCGCCGGGG 3' which binds to the transcribed mRNA of S. typhimurium,

and 5' TATTCGATGCTTTAGTGC 3' which binds to the transcribed mRNA of B. subtilis.

10. The method of claim 5, wherein the antisense oligonucleotide antibiotic binds to both a homologous sequence and a unique intergenic sequence.

11. The method of identifying bacteria, comprising the steps of:

binding a unique intergenic antisense oligonucleotide to a mRNA transcribed from a MMS operon; and

determining the amount of said binding.

12. The method of claim 11, wherein the oligonucleotide is:

5' GGCCCCGATTTTTAGCAA 3' and the bacteria is identified as E. coli.

13. The method of claim 11, wherein the oligonucleotide is:

5' CTTGCGTAAGCGCCGGGG 3' and the bacteria is identified as S. typhimurium.

14. The method of claim 11, wherein the oligonucleotide is

5' TATTCGATGCTTTAGTGC 3' and the bacteria is identified as B. subtilis.

15. An antibiotic, comprising:

at least a 10 mer oligonucleotide, wherein said oligonucleotide is complementary to a sense strand of a MMS operon and binds to a mRNA transcribed by said sense strand.

16. The antibiotic of claim 15, wherein said oligonucleotide is selected from the group consisting of:

5'GGCCCCGATTTTTAGCAA 3', 5'CTTGCGTAAGCGCCGGGG 3',

5'TATTCGATGCTTTAGTGC 3',

5'CATCCAAAGCAGTGGTAAAACTGTTT 3', and

5'TCACCGATCGGCGTTTCCA 3'.

17. The antibiotic of claim 15, further comprising:

a carrier molecule linked to said oligonucleotide, wherein said carrier molecule facilitates the uptake of said oligonucleotide into the bacterium.

18. The antibiotic of claim 17, wherein the carrier molecule is an amino acid.

19. The antibiotic of claim 15, wherein said oligonucleotide is derivatized at the 3' end to prevent degradation of said oligonucleotide.

20. The antibiotic of claim 19 wherein a phosphorothioate linked nucleotide is added to the 3' end by derivatization.

21. A method of treating bacterial infections, comprising the step of interrupting the function of proteins selected from the group consisting of S21, primase and sigma-70.

22. The method of treating bacterial infections, comprising the step of competitively inhibiting a recognition site of a protein encoded by a MMS operon by introducing a competitive oligonucleotide into a bacterium.

23. The method of claim 22, wherein a S21 recognition site is inhibited by introducing 5'GATCACCTCCTTA 3'into the bacterium.

24. The method of claim 22, wherein a primase recognition site is inhibited by introducing 5'GGCCGCCCCACATTGGGCAGGTATCTGACCAGTAGAGGGCGGCC 3' into the bacterium.

25. The method of claim 22, wherein a sigma-70 recognition site is inhibited by introducing 5' TTGACATAAATACCACTGGCGGTGATACT 3' into the bacterium.

26. The method of identifying bacteria, comprising the steps of:

treating a MMS operon to form single stranded DNA;

binding an antisense oligonucleotide to a unique intergenic sequence in the single stranded DNA of the MMS operon; and

measuring the amount of said binding.

27. The method of claim 26, wherein the oligonucleotide is:

'5 GGCCCCGATTTTTAGCAA 3' and the bacteria is identified as E. coli.

28. The method of claim 26, wherein the oligonucleotide is:

'5 CTTGCGTAAGCGCCGGGG 3' and the bacteria is identified as S. typhimurium.

29. The method of claim 26, wherein the oligonucleotide is:

'5 TATTCGATGCTTTAGTGC 3' and the bacteria is identified as B. subtilis.

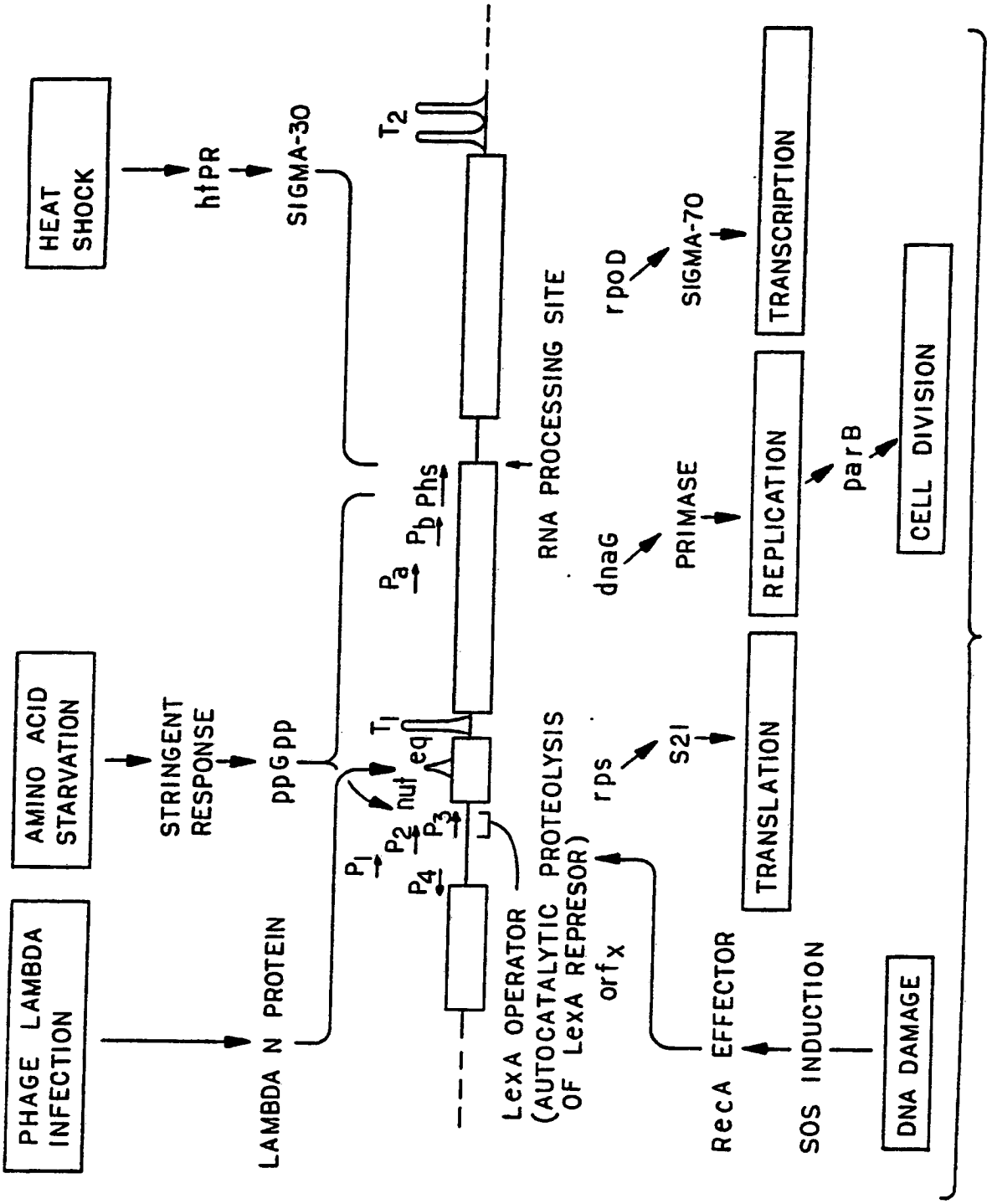


FIG. 2

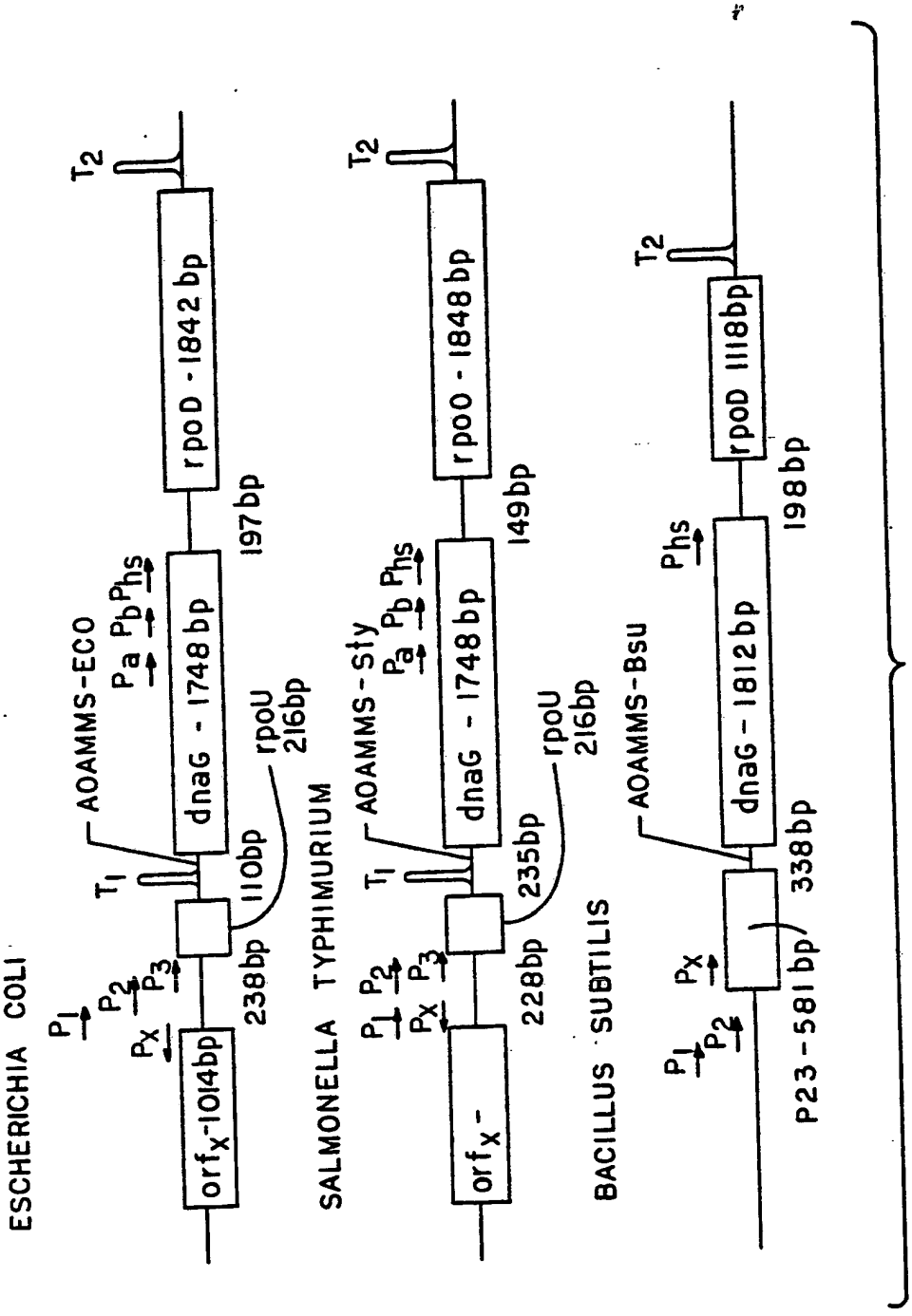


FIG. 3

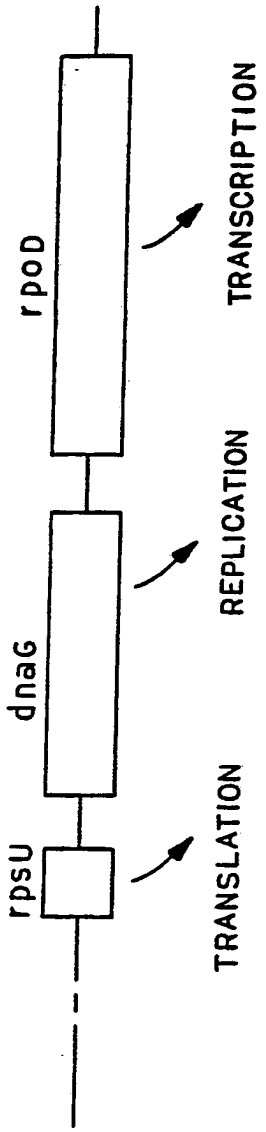


FIG. 1

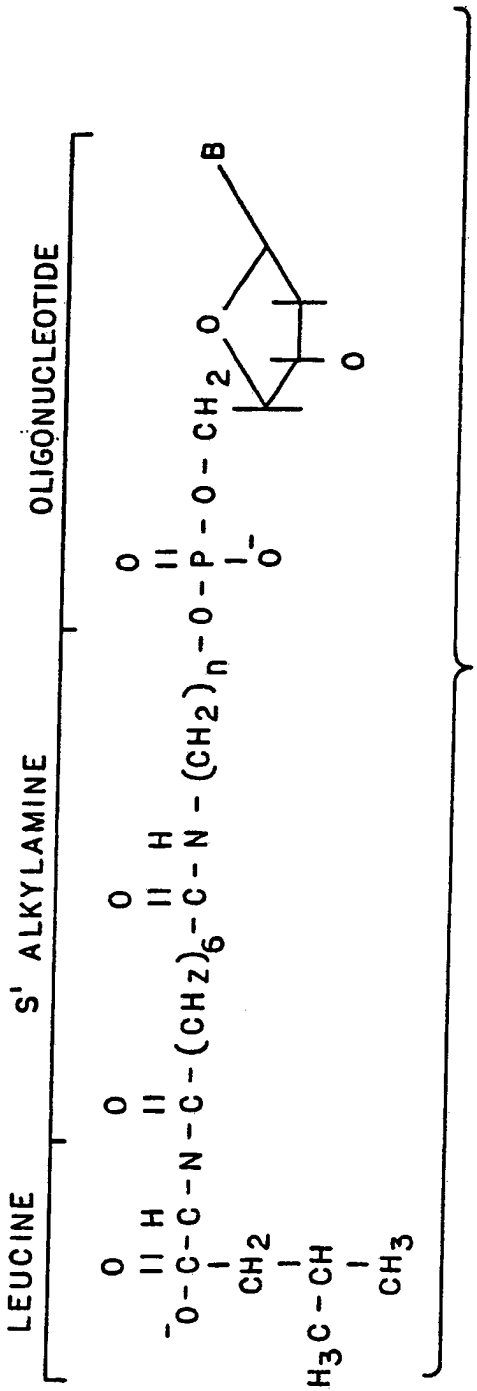


FIG. 4

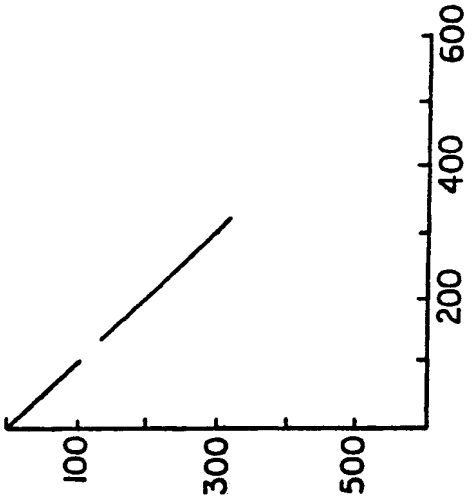


FIG. 6A

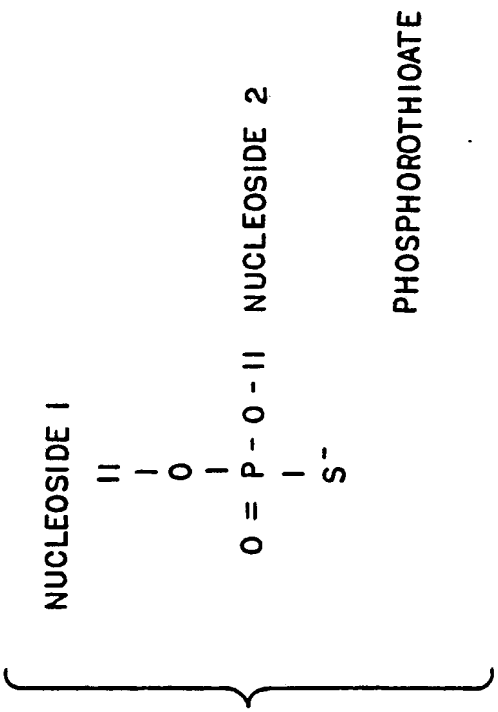


FIG. 5

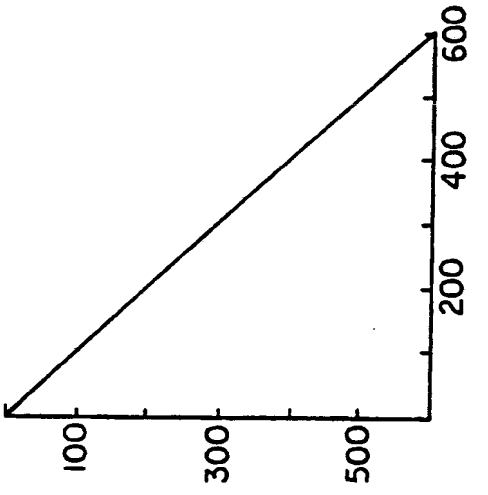


FIG. 6C

FIG. 6B

```
    E. COLI  : MAGRIPRVFINDLLARTDIVDLIDARVKLKKQGKNFHACCPFHNEKTPSFTVNGEKQFYH
S. TYPHYMURIUM : ----------V----------Y----------
    B. SUBTILIS : -GN---DEIVDQVQKSA---EV-GDY-Q-----R YFGL----G S----S-SPD--IF-

    E. COLI  : CFGCGAHGNAIDFLMNYDKLEFVETVEELAAMHNLEVPFE.AGSGPSQIERHQRQTLYQL
S. TYPHYMURIUM : ----------I-Y-.--T-L---------N----
    B. SUBTILIS : ------G--VFS--RQMEGYS-A-S-SH--DKYQIDF-DD1TVHSGARP-SSGE-KMAEA

    E. COLI  : MDGLNTFYQQSL.QQPVATSARQYLEKRGLSHEVIARFAIGFAPPGWDNVLKRFGGNPEN
S. TYPHYMURIUM : -N---D------.TH-A-KP--D--Q-----A-I-Q----------A-----N-SD-
    B. SUBTILIS : HEL-KK--HHL-1NTKEGQE-LD--LS--FTK-L-NE-Q--Y-LDS--FIT-FLVKRGFS

    E. COLI  : RQSLIDAGMLVTNDQGRSY.DRFRERVMFPIRDKRGRVIGFGGRVLGNDTPKYLNSPETD
S. TYPHYMURIUM : KAL-L-----N-E--ST-.----N----------
    B. SUBTILIS : EAQMEK--L-IRRED-SG-f---N------H-HH-A-VA-S--A--SQQ---M-----P

    E. COLI  : IFHKGRQLYGLYEAQQDNAEPNRLLVVEGYMDVVALAQYGINYAVASLGTSTTADHIQLL
S. TYPHYMURIUM : ----------YS--Q----------D--- ----------MHM-
    B. SUBTILIS : L---SKL--NF-K-RLHIRKQE-AVLF--FA--YTAVSSDVKESI-TM---L-D--VKI-

    E. COLI  : FRATNNVICCYDGDRAGRDAAWRA
S. TYPHYMURIUM : ----------
    B. SUBTILIS : R-NVEEI-L---S-K--YE-TLK-
```

FIG. 6D

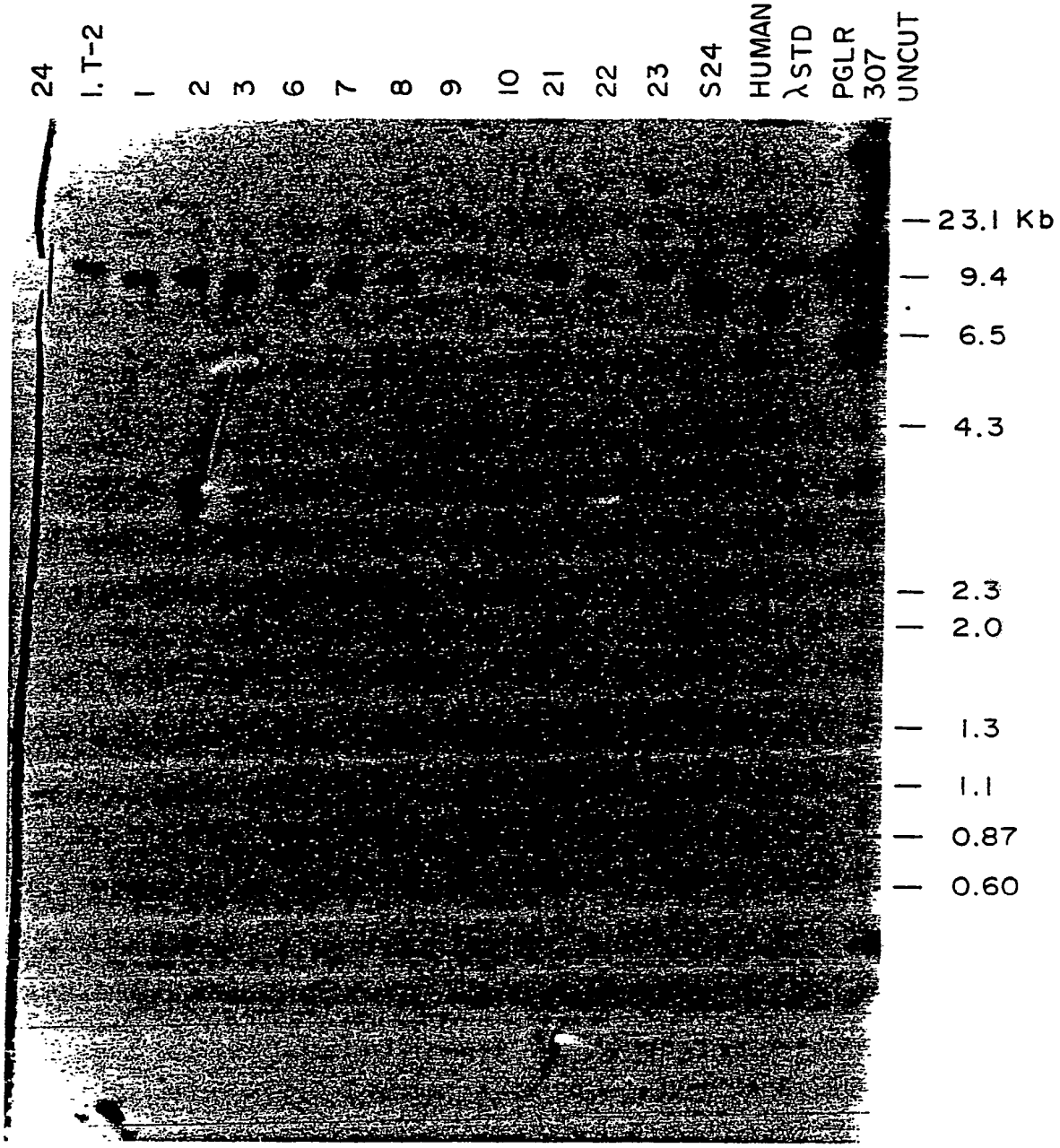


FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/02884

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C 12 Q 1/68; C 12 N 15/00; C 07 H 15/12

U.S. Cl: 435/6, 172.3; 514/44; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/6, 172.3; 935/5, 6, 8, 34, 44, 72, 78; 514/44 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Chemical Abstracts Data Base (CAS) 1967-1989; Biological Abstracts Data Base (BIOSIS) 1967-1989; MEDLINE 1967-1989. KEYWORDS: ANTISENSE, MESSAGE, MESSENGER, MRNA.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y,P	US, A, 4,801,540 (HIATT ET AL) 31 JANUARY 1989, see entire document, particularly columns 9-11.	1-29
Y	US, A, 4,740,463 (WEINBERG ET AL) 26 APRIL 1988, see entire document, particularly columns 5 and 6.	1-29
Y	US, A, 4,358,535 (FALKOW ET AL) 9 NOVEMBER 1982, see entire document, particularly columns 2-5.	11-14 & 26-29
Y	JOURNAL OF BACTERIOLOGY, Volume 169, No. 7, issued 30 June 1987 (AIBA ET AL) "Function of micF as an antisense RNA in osmoregulatory expression of the ompF gene in Escherichia coli". See entire document, particularly pages 3007-3008.	1-29

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search: 03 NOVEMBER 1989

Date of Mailing of this International Search Report: 21 NOV 1989

International Searching Authority: ISA/US

Signature of Authorized Officer: THOMAS D. MYS

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	NUCLEIC ACIDS RESEARCH, Volume 14, No. 10, issued 1986 (WANG ET AL) "Nucleotide sequence and organization of Bacillus subtilis RNA polymerase major sigma operon". See entire document, particularly pages 4293-4295.	1-29
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (USA), Volume 80, issued February 1983 (KONIGSBERG ET AL) "Evidence for use of rare codons in the dnaG gene and other regulatory genes of Escherichia coli". See entire document, particularly pages 687-689.	1-29

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . . , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . . , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	GENE, Volume 40, issued 1985, (ERICKSON ET AL) "Nucleotide sequence of the rpsU-dnaG-rpoD operon from Salmonella typhimurium and a comparison of this sequence with the homologous operon of Escherichia coli". See entire document, particularly pages 67-69.	1-29
Y	CELL, Volume 42, issued August 1985, (KIM ET AL) "Stable reduction of thymidine kinase activity in cells expressing high levels of anti-sense RNA". See entire document, particularly, pages 129-131.	1-29
Y	CELL, Volume 39, issued December 1984, (LUPSKI ET AL) "The rpsU-dnaG-rpoD macromolecular synthesis operon of E. coli". See entire document, pages 251-252.	1-29
Y	TRENDS IN BIOCHEMICAL SCIENCE, Volume 9, No. 11, issued November 1984, (LAPORTE) "Anti-sense RNA: a new mechanism for the control of gene expression". See entire document, page 463.	1-29
Y	MOLECULAR AND GENERAL GENETICS (MGG), Volume 195, issued 1984, (LUPSKI ET AL) "Promotion, termination, and anti-termination in the rpsU - dnaG-rpoD macromolecular synthesis operon of E. coli K-12". See entire document, particularly pages 391-393.	1-29
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (USA), Volume 80, issued July 1983, (LEARY ET AL) "Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots". See entire document, pages 4045-4049.	1-29